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**Abstract:** There is a need for an accessible biomarker that can complement current cerebrospinal fluid (CSF) and imaging biomarkers in an accurate and early diagnosis of Alzheimer disease (AD). Saliva is a rich source of potential biomarkers and proteins related to neurodegenerative disorders have been shown to be present in this matrix, including tau. In this study, we quantified salivary total tau (t-tau) concentration in 160 healthy elderly control (HEC), 60 mild cognitive impairment (MCI) and 54 Alzheimer's disease (AD) participants using ultra-sensitive Single molecule array (Simoa) technology. No median difference in salivary t-tau concentration was found between AD and MCI or HEC (12.3 ng/L, 9.8 ng/L and 9.6 ng/L, respectively,  $P = 0.219$ ). In addition, there was no association of salivary t-tau concentration with neurophysiological assessment or structural MRI. Despite a nominal increase in AD, due to the large overlaps in concentrations between clinical groups, we conclude that salivary t-tau is neither a suitable biomarker for AD nor for cognitive impairment.

## No association of salivary **total** tau concentration with Alzheimer's disease

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**ABSTRACT** – There is a need for an accessible biomarker that can complement current cerebrospinal fluid (CSF) and ~~positron emission tomography (PET imaging)~~ biomarkers in an accurate and early diagnosis of Alzheimer disease (AD). Saliva is a rich source of potential biomarkers and proteins related to neurodegenerative disorders have been shown to be present in this matrix, including tau. In this study, we quantified salivary total tau (~~t-tau~~) concentration in 160 ~~healthy elderly control (HEC) Alzheimer’s disease (AD), 60-68~~ mild cognitive impairment (MCI) and ~~54-53 healthy elderly control (HEC) Alzheimer’s disease (AD)~~ participants using ultra-sensitive Single molecule array (Simoa) technology. ~~No median difference in sSalivary t-tau concentration was found concentration in between~~ AD ~~did not differ from~~ MCI or HEC (~~12.3 ng/L, 9.8 ng/L and 9.6 ng/L, respectively,  $P = 0.219$~~ ), and the majority of AD cases had tau levels lower than the median in HEC. In addition, there was no association of salivary ~~t-tau~~ concentration with neurophysiological assessment or structural MRI. ~~Despite a nominal increase in AD, d~~Due to the large overlaps in concentrations between clinical groups, we conclude that salivary ~~t-tau~~ is neither a suitable biomarker for AD nor for cognitive impairment.

**INTRODUCTION** – The diagnosis of Alzheimer’s disease (AD) remains primarily reliant on clinical assessment ~~with support of structural imaging. Magnetic resonance imaging (MRI) is more widely used in AD-related investigations owing to its superior spatial resolution of regional brain atrophy and wide accessibility. Nonetheless, MRI lacks molecular specificity and as such it is downstream from the onset of molecular pathology.~~ Advancements in the latest decade have recognised the valuable role of both ~~molecular~~ imaging (~~PET Positron Emission Tomography (PET)~~ imaging of amyloid and tau aggregates) and core CSF biomarkers (A $\beta$ 42, t-tau and p-tau), which identify AD pathophysiology with high accuracy ~~at the preclinical stage~~. However, both modalities have disadvantages when considering widespread implementation of a test for suspected AD in clinical practice or for participant selection for ~~therapeutic disease modifying~~ trials. ▲

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A decrease in CSF A $\beta$ 42 is postulated to be the earliest biochemical change in AD (Palmqvist et al., 2016). However, CSF ~~total tau (t-tau)~~ may be considered more clinically relevant and a disease

intensity marker; the higher the concentration, the more intense the neurodegenerative process (Samgard et al., 2010). Using ultrasensitive measurement techniques, the protein can be measured in plasma and efforts have been made to clarify the role of plasma t-tau in AD. There is general agreement that concentrations are increased (Olsson et al., 2016) but the overlap between clinical groups is larger than for CSF tau (Zetterberg et al., 2013). In addition, the correlation of plasma with CSF tau is weak (Mattsson et al., 2016b). Plasma tau concentrations, in contrast to CSF, may be confounded by the rapid degradation of tau in blood; the half-life of tau in plasma is hours (Randall et al., 2013) compared to weeks in the CSF (Sato et al., 2018), but also by extra-cerebral tau mRNA and protein expression ([www.proteinatlas.org/ENSG00000186868](http://www.proteinatlas.org/ENSG00000186868)). It is possible that some CNS-derived proteins are eventually excreted into body fluids other than CSF and blood. The presence of tau species in saliva has been demonstrated using mass spectrometry (Shi et al., 2011). However, the relationship between salivary concentrations of t-tau and processes within the CNS is far from clear, and no conclusive data on disease association have been reported so far. In this study, we examined the diagnostic accuracy of salivary t-tau concentration for AD in dementia and mild cognitive impairment (MCI) patients as compared to healthy elderly control (HEC) individuals. Furthermore, we investigated the relationship of salivary t-tau concentration with neurophysiological and MRI measures.

**MATERIAL AND METHODS** – Saliva samples of 160 ~~HECAD~~, ~~60-68~~ MCI and ~~54-53 HEC-AD~~ participants were obtained from a single centre from the AddNeuroMed consortium (Kings Health Partners-Dementia Case Register (KHP-DCR)) (Lovestone et al., 2009). Details regarding clinical diagnosis, cognitive assessments, *APOE* genotyping and magnetic resonance imaging (MRI) acquisition have been previously described (Hye et al., 2014). Overnight fasting was required from all participants and the mouth was rinsed thoroughly 10 minutes prior to collection. Unstimulated whole saliva was collected into sterile plastic containers pre-coated with 2% sodium azide solution. Unstimulated Samples saliva was were collected into preweighed sterile plastic 30 mL containers at 30 second intervals for 2 minutes or until 2 mL had been achieved. Samples were centrifuged for 10 minutes (500xg at 4°C) placed on ice and for the removal of debris removed by a low spin (500x g for

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~~10 minutes~~) at 4°C. Aliquoted 100 µL samples (~~avoiding the pellet~~) were immediately stored at -80°C. Saliva samples were ~~thawed on ice~~, diluted 4-times and measured in duplicate for t-tau ~~concentration~~ using the commercially available Human Total Tau assay on an HD-1 Simoa instrument (Quanterix, Lexington, MA) (Randall et al., 2013). Statistical analysis was performed by using IBM SPSS Statistics, version 25 (Armonk, NY, USA). Associations between salivary t-tau and demographic factors were assessed and a generalised linear model (GLM) corrected for the significant differences of age and years of education between in the diagnostic groups (Table 1). The differences of salivary t-tau concentrations between diagnostic groups were calculated by Analysis of variance (ANOVA). Correlations between adjusted salivary t-tau levels, ~~and~~ MMSE and MRI regional measures were calculated using pearson *r*.

**CORE DATA** – The demographics for the study population are detailed in Table 1. Salivary t-tau was ~~above of the lower limit of quantification (LoQ) quantifiable~~ in 96.1% of participants included in the study (~~Supplementary Table 1~~), with an average coefficient of variation of 11.5% for duplicate measurements. Salivary t-tau was not ~~significantly~~ associated with age ( $r = 0.080$ ,  $P = 0.190$ ), years of education ( $r = -0.033$ ,  $P = 0.586$ ) ~~or~~ sex (median, 9.6 ng/L for females versus 12.3 ng/L for males;  $P = 0.872$ ) ~~or APOE ε4 genotype (median, 8.1 ng/L in non-carriers versus 9.7 ng/L in carriers;  $P = 0.788$ )~~. We observed a non-significant increase of salivary t-tau concentration across diagnostic groups (median, 9.6 ng/L for HEC, 9.8 ng/L for MCI and 12.2 ng/L for AD;  $P = 0.219$ , Fig. 1). This was also reflected by non-significant associations of increased salivary t-tau with poorer global cognitive performance as assessed with MMSE ( $r = -0.077$ ,  $P = 0.198$ ) and CDR sum of boxes (~~CDR-SoB~~) (median, 8.7 ng/L for CDR=0 and 10.2 ng/L CDR= $\leq 0.5$ ;  $P = 0.314$ ). There was no association between salivary t-tau concentration and measures of ventricular volume ( $r = -0.048$ ,  $P = 0.784$ ), hippocampal volume ( $r = 0.068$ ,  $P = 0.686$ ) ~~and~~ entorhinal cortical thickness ( $r = 0.088$ ,  $P = 0.292$ ) ~~and entorhinal cortex volume ( $r = 0.107$ ,  $P = 0.458$ )~~. However, when the AD group was analysed separately, a nominal association of lower salivary t-tau with ~~greater~~ ventricular volume was observed ( ~~$r$~~   $r = -0.492$ ,  $P = 0.045$ ).

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**DISCUSSION** – We observed no statistically significant difference in the concentrations of salivary t-tau across diagnostic groups. Additionally, we demonstrated that there was no association of salivary t-tau with MMSE and CDR-SoB-sum of boxes, although the trends observed might indicate higher salivary t-tau with poorer cognition. In a subset, MRI measures were not associated with salivary t-tau. However, in the AD group alone, larger-ventricle size was nominally associated with salivary t-tau concentration.

The only other first study examining salivary tau in AD equally reported no difference in t-tau between aged-matched controls and AD (Shi et al., 2011). However, Shi *et al* also reported an increased ratio of salivary p-tau to t-tau (Shi et al., 2011). One explanation for these contradicting results is the difference in analytical methods used. In our study, the Simoa assay employed uses a combination of antibodies that react with both normal and phosphorylated tau with epitopes in the mid- and N-terminal regions of the molecule (Mattsson et al., 2016a), making the assay specific for most tau isoforms. Shi *et al.* used a Luminex assay (based on mid-domain antibodies) that measures the phosphorylated proportion (p-tau-181) separately from t-tau and it was the p-tau/t-tau ratio that was increased in AD in their study. Moreover, Pেকেles et al using western blot found the p-tau/t-tau ratio using one specific phosphorylation site (pS396) to be increased in AD (Pেকেles et al., 2018 <https://doi.org/10.1016/j.dadm.2018.03.003>)

At present, it is hard to imagine how sampling of saliva could produce results with a clear link to changes in the brain, given the many biological barriers and compartments the marker has to cross on its way to the sampling site. Furthermore, expression of, for example, tau mRNA and protein in salivary glands and other extra-cerebral tissues such as the kidney (tau data in the Human Protein Atlas) could further limit the interpretability of t-tau measurements in saliva. Nevertheless, saliva has certain advantages over blood and CSF as a fluid for biomarker assessment. Its collection is less invasive and it is a minimally complex matrix that does not clot. Functions of the saliva are not only restricted to digestion. Saliva contains a large collection of proteins involved in the immune defence and the neuroendocrine system (Jasim et al., 2016). Further, it contains peptides that are in common

with the CSF (Jasim et al., 2016). It is also possible that the innervations of the salivary gland could provide a more direct link between the saliva and the CNS than via the blood. The submandibular gland is responsible for the vast majority of total resting and stimulated salivary volume and has been reported to be dysfunctional in AD (Ship et al., 1990). [Significantly lower levels of tau pS396 and pT231 but not t-tau has been observed in the submandibular gland of AD patients](#) (Dugger et al., 2016). Therefore, saliva could be a rich source of novel biomarkers for AD, for example lactoferrin (Carro et al., 2017), but there are major challenges in standardisation of collection and pre-processing methods ahead.

There are limitations to this study. Firstly, we are inferring that the AD patients in this study do indeed have increased tau pathology and further clarification should seek to correlate salivary t-tau concentrations with CSF or PET measures. Similar studies investigating alpha-synuclein in Parkinson's disease demonstrate no relationship between saliva and CSF concentrations (Goldman et al., 2018). Secondly, [although the largest study of salivary tau to date](#), the disproportionate numbers between the diagnostic groups could have potentially masked meaningful group differences, as we observe a non-significant median elevation in AD patients. [Lastly, we can only report the usefulness of salivary t-tau alone and that a salivary t-tau/p-tau ratio or p-tau alone should be investigated further as a potential biomarker for AD](#). In summary, ~~t-et-tau~~ is reliably measured in human saliva using the Simoa platform and exists in a range of concentrations that are not systematically different between AD and non-AD diagnostic groups. We conclude that salivary t-tau is not a reliable biomarker for AD, nor a surrogate measure of cognition or brain atrophy.

## ACKNOWLEDGEMENTS

This study represents independent research part funded by the National Institute for Health Research (NIHR) Biomedical Research Centre at South London and Maudsley NHS Foundation Trust and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.



## FINANCIAL DISCLOSURES

NJA is funded by Wallenberg Foundation and receives travel support from King's College London.

MS has received funding from the Knut och Alice Wallenberg Foundation (the Wallenberg Centre for Molecular and Translational Medicine), the Swedish Research Council, and the Swedish Alzheimer Foundation. KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. HZ has served at advisory boards of Eli Lilly and Roche Diagnostics and has received travel support from TEVA. KB has served as a consultant or at advisory boards for Alzheon, Eli Lilly, Fujirebio Europe, I.B.L. International, Novartis, and Roche Diagnostics. HZ is a Wallenberg Academy Fellow and acknowledges support from the UK Dementia Research Institute. SL is funded by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre. AH is funded by Research Centre for Mental Health and Biomedical Research Unit for Dementia.

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Mattsson, N., Zetterberg, H., Janelidze, S., Insel, P.S., Andreasson, U., Stomrud, E., Palmqvist, S., Baker, D., Tan Hehir, C.A., Jeromin, A., Hanlon, D., Song, L., Shaw, L.M., Trojanowski, J.Q., Weiner, M.W., Hansson, O., Blennow, K., Investigators, A., 2016b. Plasma tau in Alzheimer disease. *Neurology* 87(17), 1827-1835.

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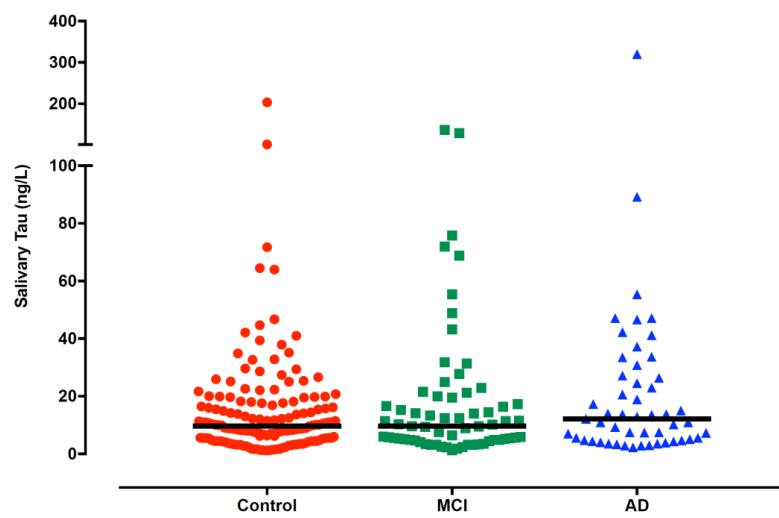
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**Table 1.** Summary of the demographic and clinical data of study participants

	Healthy Elderly Controls (n = 160)	Mild Cognitive Impairment (n = 68)	Alzheimer's disease (n = 53)	P value
Age, y (s.d)	78.0 (6.7)	79.8 (7.4)	81.4 (6.6)	0.006
Sex, male/female (% female)	66/94 (758.7)	33/35 (51.5)	23/30 (56.6)	ns
Education, y (s.d)	13.9 (3.4)	12.1 (3.3)	11.7 (2.5)	>0.001
APOE genotype (% ε4 carriers) {missing}	26 (32) {79}	9 (42.8) {47}	14 (58.3) {26}	0.041
MMSE (s.d)	28.9 (1.1)	26.8 (2.3)	22.3 (5.7)	>0.001
CDR [sum of boxes] (s.d, range)	0.15 (0.24, 0-0.5)	0.48 (0.14, 0-1)	0.89 (0.82, 0-3)	>0.001



**Figure 1.** Salivary total tau concentrations in healthy elderly controls (HEC), patients with mild cognitive impairment (MCI), and patients with Alzheimer's disease (AD).

Response to reviewers – 11<sup>th</sup> June 2018.

Reviewers' comments:

Reviewer #2: 1) container with sodium azide solution was used. It has been shown to affect measurement in a few saliva biomarkers using immunoassay. Any effect of sodium azide inclusion on the measurement of the salivary total tau using the platform in this study?

We thank the reviewer for pointing out this important feature of the article. Firstly, to address the question, we have gone back to the dental study where these samples were obtained with Professor Mark Ide (co-author). In fact, samples collected for this particular study were collected without any preservative. We apologize for this error in reporting and we have changed the manuscript to reflect this.

*“Unstimulated saliva was collected into preweighed sterile plastic 30 mL containers at 30 second intervals for 2 minutes or until 2 mL had been achieved”*

Co-authors of this paper are members of a working group for saliva standardization in the Alzheimer Association. We are therefore going to perform a side-by-side comparison on the effects of sodium azide in saliva analysis (as sodium azide has been used in saliva biomarker publications) as information for the field.

2) It is clear that total tau species had no significant changes in AD vs controls across saliva samples from various saliva studies including this.

Although there is issue regarding the reliability of assay and measurement platforms, there should not be a presumption that salivary p-tau species would turn out just as negative results as total salivary tau if they were to be measured in this study. In fact there have been consistent significant differences in p-Tau species across various biofluids including saliva. The suggestions for author would be 1) to address this as one limitation of this study as no concurrent measurement of t-Tau and p-Tau species are feasible. 2) In the last paragraph, to guide the field in presenting the issue as a future direction to work on. 3) Most importantly, since this is a negative results paper, on one hand it is wise to guide the field to avoid replication, it is equally if not more important to not dismiss the potential utility of p-Tau as a biomarker of AD just because t-Tau is not significant.

We thank the reviewer for these comments and we agree that although our data show salivary t-tau is not a biomarker for AD, no conclusions can be made on p-tau. Is this something we are going to follow up, but unfortunately cannot be performed in the same cohort. We are also going to investigate novel tau fragments that our group has been investigating in CSF. We have added an additional sentence in the discussion to reflect the comments made by the reviewer above.

*“Lastly, we can only report the usefulness of salivary t-tau alone and that a salivary t-tau/p-tau ratio or p-tau alone should be investigated as a potential biomarker for AD”*

May 18<sup>th</sup>, 2018

Re: Response to reviewers for submission to *Neurobiology of ageing*

Dear Dr. Rapp and reviewers,

Please find enclosed an edited manuscript and response to the reviewers for the entitled article, “*No association of salivary total tau concentration with Alzheimer’s disease*” by Ashton NJ *et al.*, to be submitted as a negative result to *Neurobiology of ageing*. I can confirm that all co-authors have contributed and approved the changes to the manuscript and the rebuttal.

We are very grateful for the time taken by the reviewers to generate insightful and helpful suggestions for this article. We hope that the edits to manuscript text and the explanations that accompany these edits are satisfactory to the reviewers and clarify any original concerns. We have made some minor changes to the structure of the original manuscript;

- Figure 1 has been removed from the main text and added a separate TIF file. The Figure legend remains in the manuscript text.
- Figure 1 axis label “control” has been re-labelled as “HEC” to be consist with the manuscript text
- Supplementary Table 1 has been added at the request of the reviewers.
- The reference style has been amended at the request of the editor

Sincerely yours,

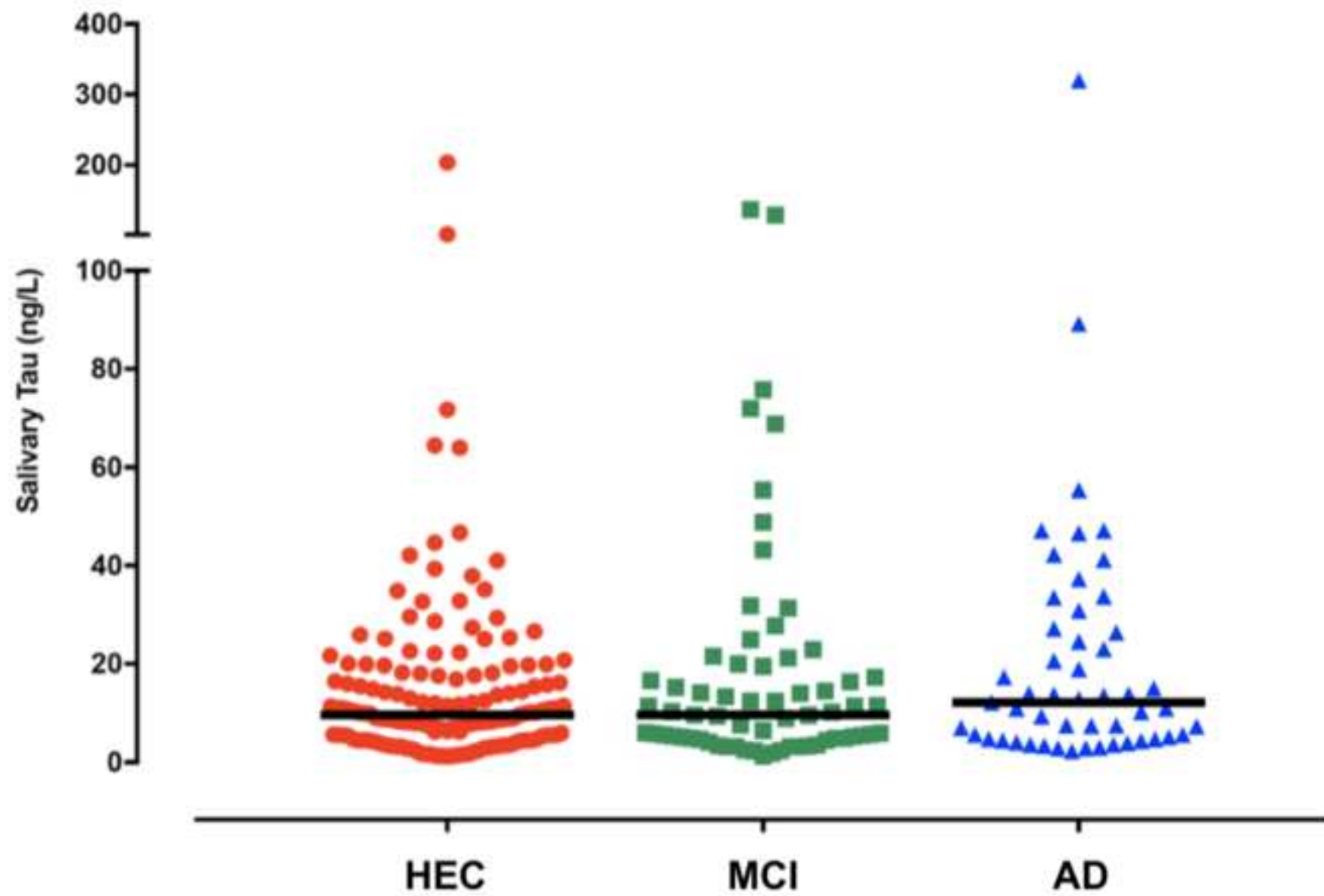
Dr. Nicholas Ashton and Professor Henrik Zetterberg on behalf of the authors

Corresponding author:

Dr. Nicholas Ashton; Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden; [nicholas.ashton@gu.se](mailto:nicholas.ashton@gu.se).



Figure(s)  
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**Verification Statement** – “*No association of salivary tau concentration with Alzheimer’s disease*” by Ashton NJ *et al.*, to be submitted as a negative result to *Neurobiology of ageing*

All co-authors have seen and approved the contents of the manuscript, and any conflict of interest is listed below.

We certify that the submission is original work and not under review elsewhere, nor has it been published previously.

KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. HZ has served at advisory boards of Eli Lilly and Roche Diagnostics and has received travel support from TEVA. KB has served as a consultant or at advisory boards for Alzheon, Eli Lilly, Fujirebio Europe, I.B.L. International, Novartis, and Roche Diagnostics.

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Sincerely yours,

Dr. Nicholas Ashton on behalf of the authors

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